Response to *Wheat dwarf virus* in wild and domesticated wheat

Genetic resources of evolutionary and environmental origins

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Cover: *Wheat dwarf virus* infested field, Uppsala, 2009. Insert, picture of leafhopper *Psammotettix alienus* (photos: Jim Nygren)

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Abstract

Wild and domesticated plants are constantly exposed to a variety of pathogens, which may trigger an arms race in evolution of defense strategies in the plant and development of virulence in the pathogen. The outcome of the interaction depends on the intensity of reciprocal selection between the interacting species, which may vary over space and time, explained as the geographic mosaic theory of coevolution. While wild crop relatives may have evolved resistance or tolerance to many pathogens, the same pathogens cause damage on crops. Studies on coevolving interactions between wild host plants and pathogens can therefore provide important knowledge for identifying genetic resources for crop improvement among wild relatives.

This PhD thesis focuses on an interesting interaction between *Wheat dwarf virus* (WDV), the leafhopper vector *Psammotettix alienus* and the wild and domesticated wheats (*Triticum* spp.). There is no known resistant wheat cultivar and severe incidences of WDV have occurred in Europe, Africa and Asia. The overall aim is to contribute to the development of improved cereal cultivars through the understanding of variation in response to WDV in wild wheat relatives (*Aegilops* spp., *Triticum* spp.), and to identify potential genetic resources. Inspired by the geographic mosaic theory of coevolution a diverse set of wild wheat relatives with different geographical, environmental, and genetic origins, directly or indirectly involved in the evolution of bread wheat was studied for response to WDV in this host plant-vector-virus interaction. Some findings are: *i)* wild wheat relatives had different response patterns during growth, including susceptibility, partial resistance and tolerance, *ii)* the response at early plant development was related to variation in onset of systemic infection and WDV accumulation in the plant, *iii)* differences in response were affected by the environment from which the wild relatives originate, *iv)* in contrast to what was expected, domestication and other genetic bottlenecks during wheat evolution have not resulted in a general increase in susceptibility in cultivated wheats, *v)* potential genetic resources were identified in *Aegilops* species carrying the D genome and of particularly interest was *Ae. tauschii*, one of the ancestors to hexaploid bread wheat. These findings can be directly applied in pre-breeding of wheat. This research also provided insights into a host-vector-virus interaction of interest for plant defense and coevolution.

Keywords: *Aegilops*, coevolution, leafhopper, partial resistance, plant-vector-virus interactions, *Psammottetix alienus*, tolerance, *Triticum*, wheat, *Wheat dwarf virus*

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It is simplicity that is difficult to make.

Berthold Brecht

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Jim Nygren**, Nadeem Shad, Anders Kvarnheden and Anna Westerbergh (2015). Variation in susceptibility to *Wheat dwarf virus* among wild and domesticated wheat. PLoS ONE 10(4), 1-24.

II **Jim Nygren**, Per-Olof Lundquist, Anders Kvarnheden and Anna Westerbergh. The wild wheat ancestor *Aegilops tauschii* - *Wheat dwarf virus* interaction: environmental origins of plant genotypes affect response patterns. Submitted.

III **Jim Nygren**, Elham Yazdkhasti, Per-Olof Lundquist, Anders Kvarnheden and Anna Westerbergh. Accumulation and spread of *Wheat dwarf virus* within host plant are delayed in the wheat D genome donor *Aegilops tauschii* compared to in the A genome donor *Triticum urartu* and bread wheat during early development. Submitted.

The contribution of Jim Nygren to the papers included in this thesis was as follows:

I Participated in planning the project, analyzing the data and writing the paper. Shared greenhouse work with one of the coauthors and carried out all laboratory work.

II Highly involved in planning the project and in analysing the data. Participated in writing the manuscript. Carried out the experiment in the greenhouse and most of the laboratory work.

III Highly involved in planning the project and in analysing the data. Participated in writing the manuscript. Shared greenhouse work with one of the coauthors and carried out most of the laboratory work.

Abbreviations

ANOVA	Analysis of variance
BYDV	*Barley yellow dwarf virus*
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
dpi	days post inoculation (=days after end of exposure)
MP	Movement protein
PCA	Principal component analysis
RSS	RNA silencing suppressor
siRNA	small interfering RNA
WDV	*Wheat dwarf virus*

1 Introduction

In both natural and human influenced habitats such as agricultural land, plants are affected by abiotic and biotic stresses. These stresses exert selection pressure on the plants and various response mechanisms may evolve, either through natural selection in genetically diversified wild populations, or through human selection in breeding of crop plants. Biotic stresses caused by pathogens may initiate an arms race between the evolution of resistance or tolerance in the host plant and the development of virulence in the pathogen (e.g. Tellier and Brown, 2007). The outcome of this coevolutionary arms race may vary across the distribution of the interacting species and over time, and the host plants may range from being highly susceptible to tolerant and resistant (Laine, 2009 and references therein). However, while the wild crop relatives may be resistant or tolerant to many pathogens, the crop plants are often highly susceptible to the same pathogens (Fisher et al., 2012, Pereira-Carvalho et al., 2015). These pathogens cause tremendous harm on the crops, which results in high yield losses as much as 25%–40% of its potential (Oerke, 2006).

Pesticides are used to lower the incidence and severity of diseases caused by pathogens. However, these chemicals are of environmental, ecological and human health concerns due to their pollution of water and soil, and will therefore also be economically costly for the whole society. Moreover, repeated use of pesticides may evolve resistance to pesticides in the pathogen (Bass et al., 2014). The use of other methods and practices in agriculture are therefore crucial for reducing the harm of pathogens. Cultivation practices such as crop rotation, intercropping of multiple crops within the same field and change in sowing time to reduce the exposure to the pathogen are methods used in different agricultural regions (Lindblad and Waern, 2002, Jones, 2006, Pappu et al., 2009, Boudreau, 2013). For insect vector-transmitted pathogens such as viruses biological pest control using microbial pesticides or pheromones may be applicable (Mendoza-Figueroa et al., 2014, Bruce et al.,

2015). Cultivation of high-yielding resistant cultivars in combination with relevant cultivation practices may, however, be the most effective and economic way to reduce the use of pesticides and to obtain an ecological and sustainable agriculture. The agriculture is further challenged by global climate change and the incidence and severity of diseases may increase with the predicted increase in temperature and changes in precipitation (Roos et al., 2011). Breeding of resistant crops is therefore considered to be the most effective and economic way to reduce yield losses caused by pathogens (Lecoq et al., 2004).

Breeding of improved cultivars is dependent on genetic and phenotypic diversity in the breeding population and appropriate genetic resources. All living material with genes and traits of present or potential value for humans are considered as genetic resources by the Convention of Biological Diversity (Cbd.int, 2016). The wild relatives of crops harbor large genetic and phenotypic diversity as a result of various direction and intensity of selection across different environments and geographical regions. Due to the strong natural and human selection during the domestication process the genetic diversity was reduced in the domesticated crop and much of the traits and genes were left behind in the wild relatives (e.g. Doebley et al., 2006, Olsen and Wendel, 2013). The wild relatives are therefore potential genetic resources for development of resistance and tolerance to pathogens in future improved cultivars.

Knowledge about interactions between viruses and their wild host plants are limited compared to wild plant-fungal interactions (Mauck et al., 2012, Prendeville et al., 2012). For a better understanding of the variation in response in wild crop relatives to virus infections we have focused on an interesting interaction between *Wheat dwarf virus* (WDV), the leafhopper vector *Psammotettix alienus* and the wild and domesticated wheats. Severe incidences of WDV and yield losses have been reported from several parts of Europe, Africa, Western Asia and China (Benkovics et al., 2010, Figure 1). Although some variation in susceptibility occurs among bread wheat cultivars (Vacke and Cibulka, 2001, Lindblad and Waern, 2002, Širlová et al., 2005, Benkovics et al., 2010), there are no known resistant cultivars, and the wild wheat relatives may be the only sources of improvement of WDV resistance in wheat.

The overall aim of this thesis is to contribute to the development of improved cereal cultivars through the understanding of variation in response to virus infections in wild wheat relatives from different environmental and evolutionary origins, and to identify potential genetic resources. This research, called pre-breeding, is part of the activities necessary before effective and well designed plant breeding programs can be developed. Plant breeding plays a key

role in the improvement of sustainable agriculture and food security. This thesis will therefore add a part of knowledge that can assist in meeting the challenge of the increasing demand for food by a rapidly growing global population.

Figure 1. WDV infected winter wheat field in Björklinge, Uppsala, Sweden, in 2010.

1.1 Domestication of crops

The early farmers found that burning of native forest favored the growth of colonizing edible plants. Seeds from colonizing plants with desirable traits were selected and gathered by the farmers and re-sown in their fields. This early agricultural practice was repeated over time and the phenotype of the plants changed, resulting in crop plants with increased seed number and/or size, increased apical dominance and fewer stems, loss of seed shattering and seed dormancy (Doebley et al., 2006, Fuller, 2007, Matsuoka, 2011, Peng et al., 2011). Due to the strong selection during domestication much of the genetic diversity was unconsciously left behind in the wild crop populations (e.g. McLauchlan et al., 2001, Haudry et al., 2007). This may also have been the case for traits not directly selected for such as resistance and tolerance to pest and diseases. Thus, the genetic bottleneck caused by domestication has

hampered the reciprocal selection and the arms race between the pathogen and the host plant, and thereby increased the susceptibility in the crop.

Besides the high susceptibility to pathogens the drastic change in growing conditions for cultivated crops compared to their wild relatives has further increased the incidence of diseases in crop fields. Wild relatives of crops are adapted to a wide range of habitats with various abiotic and biotic stresses as a result of natural selection, and thus show large genetic and phenotypic variation throughout their distribution. They grow in communities with high species diversity and multi-species interactions. On the contrary, the complexity and diversity have consciously been diminished in agriculture. Dense populations of crops with low genetic diversity are cultivated in homogenous habitats influenced by human activities (Zohary, 2004, Jones, 2009, Pagán et al., 2012). The aggregation of crop plants in dense fields increases transmission of non-vector as well as vector transmitted pathogens (Burdon and Chilvers, 1982, Thresh, 1982, Roy, 1993, Lively et al., 1995). It will allow longer feeding sessions for insect vectors, which may increase transmission rates and accumulation of viruses and other pathogens (Power, 1991, Stukenbrock and McDonald, 2008). During domestication pathogens and their insect vectors may have coevolved and simultaneously adapted to new host plants (Stukenbrock et al., 2007).

1.2 Domesticated wheats

1.2.1 Origin

Cultivated einkorn (*T. monococcum* ssp. *monococcum*) is the first domesticated wheat species. It originates from wild einkorn (*T. monococcum* ssp. *boeticum*) and the domestication took place about 10,000 - 12,000 years ago in the Karacadag mountain range in southeastern Turkey, which is part of the Fertile Crescent region in West Asia (Heun et al., 1997, Ozkan et al., 2005, Luo et al., 2007). Many major crops such as cereals and legumes have been domesticated in this region (Doebley et al., 2006). Within the same region, the wild diploid *T. urartu* hybridized with the wild wheat species *Ae. speltoides* (Luo et al., 2015a). *T. urartu* is the A genome donor (Dvořák et al., 1993, Dvorak et al., 1998, Zhang et al., 2006, Odintsova et al., 2008) and *Ae. speltoides* is the putative B genome donor of hexaploid wheat (Adonina et al., 2005, Kilian et al., 2007, Salse et al., 2008, Adderley and Sun, 2014). The natural hybridization and subsequent polyploidization events took place about 200 000 years ago and formed the tetraploid wild emmer wheat (*T. turgidum* ssp. *dicoccocon*) carrying the A and B genomes (Dvorak and Akhunov, 2005). Wild emmer was then domesticated into cultivated emmer (*T. turgidum* ssp.

dicoccocon) in the southern areas of the Fertile Crescent (Civáň et al., 2013). Durum wheat (*T. turgidum* ssp. *durum*), which is also tetraploid, is alleged to have evolved from cultivated emmer in the eastern parts of the Mediterranean (Salamini et al., 2002, Luo et al., 2007).

Cultivated emmer wheat migrated eastward from its domestication site about 7 000 to 8 000 years ago to Transcaucasia and Caspian Iran. In the same region the wild relative and D genome donor of hexaploid wheats, *Ae. tauschii*, has its origin and largest genetic diversity (Dvorak et al., 1998, Saeidi et al., 2006, Matsuoka et al., 2008a, Saeidi et al., 2008, Dvorak et al., 2012, Wang et al., 2013). The overlapping distribution of cultivated emmer and *Ae. tauschii* resulted in hybridization between these species and subsequent polyploidization gave rise to the first hexaploid wheat carrying the A, B and D genomes. It is debated whether hexaploid wheat has a single origin or if it has evolved in more than one site (Caldwell et al., 2004, Giles and Brown, 2006, Dvorak et al., 2012, Jones et al., 2013, Wang et al., 2013).

An important trait that underwent changes and that have been used to trace events of domestication is the hull character. The hull character is found in *Ae. tauschii* and in the four wild species of wheat and in three domesticated varieties: einkorn, emmer and spelt (Salamini et al., 2002). Only durum wheat and bread wheat are free-threshing or naked forms. It has been proposed that bread wheat evolved from cultivated emmer but with introgression interferences from wild emmer when hybridizing with *Ae. tauschii* (Dvorak et al., 2006). It have also been suggested that durum and not emmer wheat is the tetraploid progenitor to bread wheat since they in present-time coexist in areas of south Caspian Iran (Matsuoka et al., 2008a). Dvorák *et al.* (2012) have suggested that the early hexaploid wheat was not free-threshing as commonly accepted but hulled, and emerged from a free-threshing tetraploid wheat. The hulling, or glume tenacity, was lost through a mutation in the *Tg* locus in this early hexaploid wheat form.

1.2.2 Loss of genetic diversity

The domesticated wheats, durum, emmer, and bread wheat show lower genetic diversity than their ancestor wild emmer wheat (Haudry et al., 2007). The genetic diversity in wheats was reduced in several initial selective events during domestication. Even though the natural hybridization of the BA and D genomes into hexaploid wheat resulted in additional genomes within each plant, the total genetic diversity within the crop was reduced. The loss of genetic diversity in the evolution of bread wheat may be explained by two selective events; *i*) during the natural hybridization and subsequent polyploidization limited number of genotypes from the wild ancestral

populations were involved and *ii*) during the domestication the early farmers used a limited number of plants from the ancestral population and repeatedly over plant generations collected seeds only from the plants with favourable traits (Dvorak et al., 1998, Dubcovsky and Dvorak, 2007, Naghavi et al., 2008, Mizuno et al., 2010, Naghavi et al., 2010, Tahernezhad et al., 2010, Jones et al., 2013). The hybridization, polyploidization and domestication bottlenecks were particularly affecting the D genome of bread wheat (Caldwell et al., 2004, Reif et al., 2005, Naghavi et al., 2009, Naghavi et al., 2010).

1.3 Wild relatives of wheat

1.3.1 Classification

Like domesticated wheats all species of the wild relatives are annuals. They are divided into three genera (family *Poaceae*), *Aegilops* with 22 species, *Triticum* with four species and *Amblyopyrum* with only one species, according to the widely accepted classification system by van Slageren (1994). These species have three different ploidy levels (diploid, tetraploid and hexaploid). Each diploid genome carries 7 chromosomes in each haploid set. Thirteen of the species are diploids carrying the A, C, D, M, N, S, T or the U genome and the remaining are tetraploids and hexaploids with different combinations of the genomes (Waines and Barnhardt, 1992, Kilian et al., 2011).

1.3.2 Origin and distribution

The diploid species of *Aegilops* originate from Transcaucasia (border of Eastern Europe and Southwest Asia), from where they migrated both west- and eastward into the Mediterranean and Central Asia (Hammer, 1980, Matsuoka et al., 2008b, Takumi et al., 2009, Dudnikov, 2012). During the migration diploid *Aegilops* species hybridized and formed allopolyploids (Meimberg et al., 2009). The distribution of the wild relatives expanded with the increase in genome ploidy number (Villar et al., 1998, Feldman et al., 2012). The current native distribution ranges from the Mediterranean region and North Africa in the west to China in the east, and from the southern Russia in the north to northern Pakistan and India in the south (Zohary et al., 2012). Their distribution is restricted by inaccessible environments such as the deserts of the Arabian Peninsula and by the Tian Shan Mountains in central Asia (van Slageren, 1994). The largest species diversity of the wild relatives has been observed in the region between western Syria and northeast Lebanon and in northern Iraq within the Fertile Crescent.

The wild relatives, in particular the allopolyploid species of *Aegilops*, are growing in diverse habitats with large variation in temperature, precipitation,

humidity and frost frequency, such as temperate grasslands, steppes and savannas, conifer and mixed forests and subalpine grasslands (van Slageren, 1994, Jones et al., 2013). However, the diploid *Ae. tauschii* and D genome donor of bread wheat, is adapted to variable climate conditions and can be found in the dry Artemisia steppes and desert margins, the hot plains of southern Iran, the continental climate of the Central Asian steppes eastward to western China, and the hilly and rain-soaked temperate forest belt of the southern coast of the Caspian Sea (Zohary et al., 1969, Tanaka and Tsujimoto, 1991, Dudnikov, 2009). It is typically found in communitites with other *Aegilops* species such as *Ae. biuncialis*, *Ae. crassa*, *Ae. neglecta* and *Ae. triuncialis*, and grasses within the genera *Bromus* and *Phleum* (Yen et al., 1983, van Slageren, 1994).

1.3.3 Morphological and genetic diversity

The adaptability to various growing conditions under different selection pressure has shaped the phenotypic diversity and genetic structure of the wild relatives and they show large morphological and genetic diversity both among and within species (Hegde et al., 2000, Mizuno et al., 2010, Sohail et al., 2012). They differ in height, number of leaves and tillers, spike length and seed weight (Khan and Tsunoda, 1970, Villar et al., 1998, Arzani et al., 2005, Villar et al., 2005, Karagöz et al., 2007). *Ae. tauschii* is the fastest growing wild relative (Villar et al., 1998), and exhibit rapid leaf expansion rates in the early growth stages, similar to bread wheat (Bultynck et al., 2004). The extent of morphological and genetic diversity may, however, not necessarily be related to the size of the species range. For example, the diploid *Ae. sharonensis*, has as large morphological and genetic diversity as the wild relatives which have a much broader geographic distribution across variable environments (Olivera and Steffenson, 2009, Olivera et al., 2010).

1.3.4 Genetic resources

The extensive phenotypic and genetic diversity of the wild wheat relatives across their range make them potential genetic resources for improvement of wheat cultivars. In contrast to the homogenous monoculture fields, the large diversity of the wild populations and the pathogen enables an arms race between these species, and the host population may reach a stable and balanced polymorphism, resulting in a variation in host plant response. This is caused by negative-frequency dependent selection, meaning that when a phenotype such as resistance is rare in the population, the phenotype is relatively favored by natural selection but when it becomes more common, the fitness decreases and the interaction has reached equilibrium. The expected variation in response

emphasises the importance to search for resistant or tolerant genotypes among the wild relatives. The widespread natural populations of wild relatives of wheat found in the steppes and meadows of central Asia and Caucasus may provide novel genetic resources for cereal improvement (Stolton et al., 2006). In fact, genotypes (accessions) of the wild wheat relatives have been used as genetic resources for improvement of bread wheat to fungi and other pathogens (e.g. Millet, 2007, Schneider et al., 2008). For example, the three genome donors to hexaploid wheat, *T. urartu*, *Ae. tauschii* and *Ae. speltoides* are carrying genes of resistance to rust and powdery mildew (Schneider et al., 2008, Huang et al., 2009, Rouse and Jin, 2011, Vikas et al., 2014). In addition, variation in resistance has been found in *Ae. tauschii* and *T. monococcum* to *Soil-borne wheat mosaic virus* (SBWMV) (Kanyuka et al., 2004, Ward et al., 2005, Hall et al., 2009), and in *Ae. geniculata* to *Barley yellow dwarf virus* (BYDV) (Zaharieva et al., 2001). In a less recent study *Ae. caudata*, *Ae. ovata* and *Ae. triuncialis* were found to show mild symptoms to WDV infection in comparison to spring wheat (Vacke, 1972). However, the wild relatives of wheat remain a largely untapped reservoir of genetic and phenotypic diversity and are of high interest in breeding of resistance and tolerance in cultivated wheats (Valkoun, 2001, Feuillet et al., 2008, Ordon et al., 2009).

1.4 *Wheat dwarf virus*

1.4.1 Genome structure and diversity

WDV, the pathogen in the tripartite interaction studied in this PhD thesis, belongs to the genus *Mastrevirus* and the family *Geminiviridae*. It has a single-stranded (ss) circular monopartite (single DNA molecule) genome (Lindsten et al., 1980, Muhire et al., 2013). Like all mastreviruses the genome size of WDV is small. It consists of approximately 2750 nucleotides and encodes four different proteins (MacDowell et al., 1985, Köklü et al., 2007). The proteins Rep and RepA, translated from a spliced transcript, are associated with the replication of the virus and therefore expressed early during the infection (Schalk et al., 1989, Boulton, 2002). Later during the infection the coat protein involved in encapsidation of the virus, and the movement protein (MP) active during the cell-to-cell transport within the plant, are expressed.

WDV has been isolated from both wheat and barley, however, wheat-infecting isolates of WDV are usually unable to infect barley and the other way around (Lindsten and Vacke, 1991, Kvarnheden et al., 2002, Köklü et al., 2007). Based on genome sequences, three WDV strains, named WDV-A, WDV-B and WDV-D, have been identified on barley and two strains, WDV-C and WDV-E, on wheat (Muhire et al., 2013). Most of the WDV isolates from

wheat in Europe and Asia share a high genome sequence identity and form the strain WDV-E. However, a large genetic diversity in the wheat-infecting isolates has been found to be concentrated in some regions of the WDV genome including introns, short and long intergenic regions and the coding region of the replication-association protein Rep A (Wu et al., 2008). The genetic variation within WDV and the other interacting species, the vector and host plant, is fundamental for their coevolutionary relationship, however, if the variation found in WDV is correlated to the virulence is not yet known.

1.4.2 Virus infection

Even though processes such as transcript splicing increase the coding potential of the small genome, WDV is also dependent on host plant proteins for replication and movement within the plant. Within the infected cell nuclei, the virus manipulates the cell cycle of the host plant and alters the cell gene expression to create multiple new copies of itself (See review by Hanley-Bowdoin et al., 2013 and references therein). Viruses systemically infect plants by virus multiplication in the plant cells, virus movement to adjacent cells through plasmodesmata and long-distance movement within the plant through the phloem. In the phloem, the virus is transported from the initially infected leaves (lower, fully expanded leaves) throughout the plant to the roots, the upper younger leaves, and the grains (Vuorinen et al., 2011, Hipper et al., 2013). Inhibition at any of these steps by active defence responses or by incompatible interactions between viral and host factors can lead to virus resistance (Gómez et al., 2009, García and Pallás, 2015). Virus-encoded MPs interact with host plant proteins to promote cell-to-cell and long-distance movement within the plant. The highest concentrations of virus are typically found in the youngest and most rapidly growing tissues and the lowest content in the older leaves with less metabolic activity and dividing cells (Ber et al., 1990, Zamir and Czosnek, 1994, Sadeghi et al., 2010, Drechsler, 2011).

1.4.3 Host range of WDV

Viruses are completely dependent on their hosts and they are specifically adapted to certain hosts. WDV may be considered as a grass generalist pathogen since its host range encompasses not only wheat but also other cereals such as barley, oat and rye. Moreover, it can infect several wild grasses such as *Aegilops* ssp., *Avena* ssp., *Bromus* ssp., *Hordeum* ssp., *Lolium* ssp., and *Triticum* ssp. (Vacke, 1972, Mehner et al., 2003, Nygren et al., 2015). For infection of wheat, wild grasses are of less importance as primary sources compared to cultivated wheat. However, the grasses growing in vicinity to cultivated cereal fields may act as reservoirs of WDV (Ramsell et al., 2008).

1.5 Host plant responses

1.5.1 Tolerance and resistance

In host plants, the response to virus infection can vary greatly. They may either die prematurely from the disease or continue to live throughout their normal life span, but with different degrees of symptoms and virus content. The variation in symptoms and virus accumulation levels reflects different evolutionary plant defense strategies to virus infection such as tolerance and resistance (Miller et al., 2005, Best et al., 2008). The host plant may accept and tolerate the presence of the intrusive virus and allow it to replicate and accumulate to high levels and simultaneously only develop few visible symptoms of disease (Little et al., 2010). This is a common strategy in wild plant populations (Miller et al., 2006, Best et al., 2008, Paper I-II). An alternative strategy is to repress viral multiplication and spread to minimize the damage incurred by the infection (Roy and Kirchner, 2000, Miller et al., 2005). These traits are heritable and wild plant populations maintain many forms of susceptible, tolerant and resistant plants (Best et al., 2008). The visible response to infection by WDV has been shown to vary between different host plants, as seen in the wild relatives of wheat (Paper I–III). Infection of wheat plants at an early developmental stage generally induces severe leaf chlorosis and stunting of vegetative and reproductive tillers (Vacke, 1972, Lindblad and Sigvald, 2004, Figure 2).

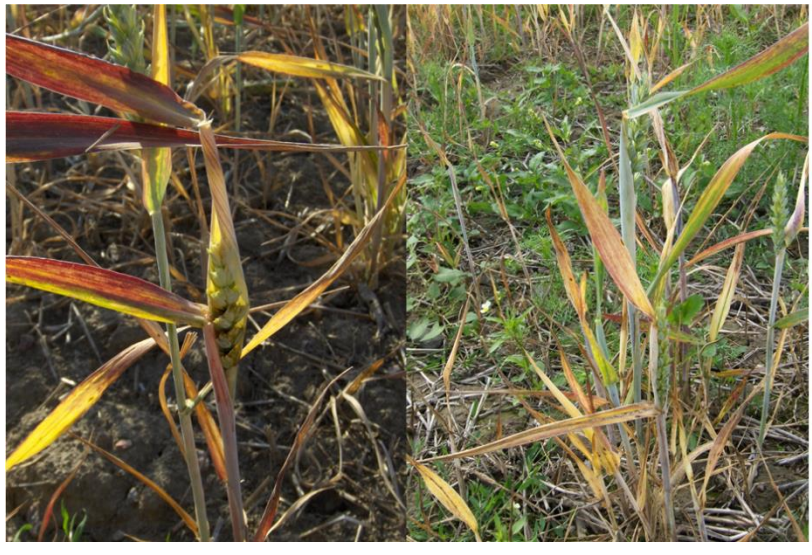

Figure 2. WDV infected plants of winter wheat. Left: Plant with stunted spike and chlorotic and discolored leaves. Right: Dwarfed plants with chlorotic and discolored leaves.

Geminivirus-infected plants can recover from the disease (Bengyella et al., 2015), and may be seen as an alternative tolerance strategy. In such plants a progressive remission or disappearance of symptoms occurs over time after initially being severe and systemic. Newly developed leaves emerge asymptomatic in plants although the virus infection persists and never completely clears from the host. In this way, the plant is displaying tolerance to virus infection. Symptom recovery during infection by RNA viruses is often accompanied by reduced virus titres (Ma et al., 2014, Nie and Molen, 2015), and this has been found also for geminivirus infections of *Nicotiana benthamiana*, cassava (*Manihot esculenta*), cantaloupe (*Cucumis melo*), pepper (*Capsicum annum*) and watermelon (*Citrullus lunatus*) (Chellappan et al., 2004, Carrillo-Tripp et al., 2007, Hagen et al., 2008, Rodríguez-Negrete et al., 2009).

1.5.2 Defense and counter-defense

The development of plant symptoms and a possible subsequent recovery are the outcome of infection, plant anti-viral defence and viral counter-defence. The differences in strength between plant defence and virus may cause different outcomes in virus accumulation and degree of symptoms. The RNA silencing system is a central defence against plant viruses that is activated by intruding double-stranded RNA. To repress virus multiplication, the RNA silencing acts both on transcriptional and posttranscriptional levels (Raja et al., 2008, Hohn and Vazquez, 2011, Pumplin and Voinnet, 2013, Incarbone and Dunoyer, 2013, Ghoshal and Sanfaçon, 2015, Zhao et al., 2016). Transcriptional gene silencing acts by methylation of the genome of DNA viruses whereas post-transcriptional gene silencing represses production of viral proteins by degradation or translation repression of viral RNA. Viruses can, however, counteract the RNA silencing defences by producing silencing suppressor (RSS) proteins, which act through diverse mechanisms at different steps of RNA silencing (Csorba et al., 2015). Several RSS proteins have been identified for geminiviruses (Hohn and Vazquez, 2011, Csorba et al., 2015), and recently the Rep and RepA proteins of WDV as well as RepA of the closely related *Oat dwarf virus* have been demonstrated to be able to suppress RNA silencing (Liu et al., 2014, Wang et al., 2014a, Qian et al., 2015).

The production of viral RSS proteins also results in symptom development of the host plant. MicroRNA molecules are regulating different processes in the plant by RNA silencing such as development. The interference of viral RSS proteins with the RNA silencing will result in reduced growth and leaf chlorosis (Chapman et al., 2004, Hanley-Bowdoin et al., 2013, García and Pallás, 2015). It was shown that the coat protein of the *Cucumber mosaic virus*

(CMV; family *Bromoviridae*) and the encoded RNA silencing suppressor 2b protein causes leaf chlorosis by repressing the expression of chloroplast and photosynthesis related genes in tobacco (Mochizuki et al., 2014). A decrease of photosynthesis may subsequently initiate other processes involved in the host plant defense such as respiration (Berger et al., 2007). However, during infection, all virus proteins may potentially interfere with processes in plants, not only RSS proteins, sometimes leading to visible effects on the plant phenotypes (García and Pallás, 2015). Geminiviruses reprogram many plant processes during infection, often related to hormone signalling (Hanley-Bowdoin et al., 2013).

Symptom recovery usually correlates with a decrease of virus levels and an increase in accumulation of viral siRNA (Chellappan et al., 2004, Rodríguez-Negrete et al., 2009, Bengyella et al., 2015). Plant recovery from infection by a mutant of *Beet curly top virus* (family *Geminiviridae*; genus *Curtovirus*) was shown to be a consequence of RNA silencing repressing virus accumulation and thereby attenuating the effects of the virus (Raja et al., 2008). Symptom recovery was also found in pepper plants infected with *Pepper golden mosaic virus* (family *Geminiviridae*; genus *Begomovirus*) and differences in the structure of minichromosomes were found in symptomatic and recovered asymptomatic plant tissues (Ceniceros-Ojeda et al., 2016). Minichromosomes isolated from symptomatic tissue had a low level of DNA methylation, while minichromosomes from recovered tissue had a high level of DNA methylation, indicating transcriptional gene silencing inactivation of the virus.

1.5.3 Controlled by single or many genes

Even though tolerance is a common defense strategy in wild plants very little is known about its genetic basis. However, much more is known about the genetic control of resistance to pathogens, in particularly, to fungi. There are different types of plant resistance to pathogens showing different genetic basis. Qualitative resistance is controlled by single genes of large effect on the phenotype and may be either dominant or recessive (Maule et al., 2007). This type of defense often gives resistance to similar genotypes (races and strains), and is therefore called specific resistance. Quantitative resistance is on the other hand controlled by many genes of small to larger individual effects and could be both dominant and recessive. It is conferring resistance to several races and strains, and called non-specific resistance or partial resistance. Dominant resistance genes often operate through gene-for-gene recognition, meaning that for each gene which is controlling resistance in the host plant there is a matching gene which controls avirulence in the virus. A host plant which is producing a specific resistance gene product is thus resistant to a

pathogen which produces the corresponding avirulence gene product. In contrast, recessive resistance genes are coding for the lack of production of specific host factors necessary for the pathogen to complete its infection cycle. When host factors are missing the host plant are resistant to the pathogen.

Knowledge about the genetic basis of plant response to geminiviruses including WDV is limited. However, in wild tomato five genes and two alleles of one of the genes have been identified for resistance to *Tomato yellow leaf curl virus* (Butterbach et al., 2014). These wild genes have been introgressed into cultivated tomato to improve resistance (Verlaan et al., 2013, Butterbach et al., 2014, Prasanna et al., 2015). These studies demonstrate the usefulness of wild relatives as sources for identification of resistance and subsequent breeding.

1.6 Leafhopper *Psammotettix alienus*

1.6.1 Transmission of WDV

Like many viruses, WDV is dependent on insect vectors for transmission between plants (Vacke, 1961, Lindsten et al., 1970, Hogenhout et al., 2008). The main vector is the leafhopper *Psammotettix alienus* (order Hemiptera, family Cicadellidae) (Vacke, 1961, Wang et al., 2014b) (Figure 3). However, a related species, *P. provincialis*, has been reported to be able to transmit WDV to wheat plants (Ekzayez et al., 2015).

Figure 3. Leafhopper *Psammotettix alienus*. Left: Adult leafhopper, Right: Nymph.

P. alienus acquires WDV through the phloem sap when penetrating the leaf with its stylet for feeding (Fereres and Moreno, 2009). The virus is then circulated in the intestine and haemolymph of the leafhopper without replication until it is released in the salivary glands. In the glands the virus is mixed with the saliva and inserted into the host plant during the next feeding bout. The virus may also directly pass to the salivary glands enabling a non-viruliferous leafhopper to acquire and transmit the virus within only 15 minutes (Wang et al., 2014b). *P. alienus* is viruliferous and able to transmit WDV even after ten visits and transfers to new uninfected host plants without acquisition of WDV in between the visits (Lindsten et al., 1970). The persistence of WDV transmission is proposed to be a result of virus accumulation in the intestine of the leafhopper and the gradual release to the salivary glands. *P. alienus* is able to acquire and transmit WDV in all larvae (nymph) stages and adult ages (Vacke, 1964, Mehner et al., 2003). However, transmission efficiency decline with increasing age and is suggested to be caused by the destruction of the phloem by the larger stylet of the adult leafhoppers during feeding (Stafford et al., 2012).

Figure 4. Plants of the winter wheat cultivar Kosack 42 days after the end of treatments. Left: non-exposed plant. Middle: plant exposed to WDV-free *P. alienus* leafhoppers. Right: plant exposed to WDV viruliferous leafhoppers. The trial was conducted in a greenhouse and during the treatment each plant was separately kept in a cage covered by nylon fabric. At the three-leaf stage twelve plants were each exposed to five WDV-free leafhoppers, six plants were each exposed to five viruliferous leafhoppers and six plants were not exposed. The insects and cages were removed after seven days of exposure. Information about the growing conditions and the host-vector-virus system used in this and trials conducted in paper I-III are presented in section Materials and Methods.

1.6.2 Effects of WDV free leafhoppers

Only a few studies have been investigating the effect of the feeding *per se* on host plants without virus transmission and contrasting results have been found (Lindsten et al., 1970, Watanabe and Kitagawa, 2000, Luo et al., 2015b). Thus, we found it important to evaluate the effect of *P. alienus* in our own experimental system used for investigation of plant response to WDV infections. We compared the response of the cultivar Kosack, which is known to be susceptible to WDV, in three different treatments. In one of the treatments the plants were exposed to WDV viruliferous leafhoppers, in another treatment to WDV free leafhoppers, and in the third treatment they were not exposed to any leafhoppers (Figure 4). The plants were studied for percentage of leaf chlorosis (yellowing of leaf) at 28 and 56 days after end of exposure and number of leaves 56 days after end of exposure. Plants exposed to WDV free leafhoppers were not significantly different from the non-exposed plants at both 28 dpi, and at 56 dpi, whereas the plants exposed to viruliferous leafhoppers had significantly higher degree of leaf chlorosis at 28 dpi ($p<0.001$, one-way ANOVA). At 56 dpi none of the plants exposed to viruliferous leafhoppers were alive. Even though we did not find any evidence for symptoms caused by virus free feeding leafhoppers, more intensified exposure of larger populations of *P. alienus* on winter wheat in the field, may cause damages on the host plant. Moreover, the feeding by *P. alienus* may introduce changes in the nutritional qualities of the plant which may affect the fitness and behaviour of other insects sharing the same host plants as *P. alienus* (Luo et al., 2015b).

1.6.3 Distribution

Some leafhoppers such as *P. alienus* are generalist feeders on grasses. These leafhoppers are found in both large permanent grassland habitats such as the prairies in North America and Canada, and grasslands and savannas in Africa, and in small patchy grass vegetation within the conifer and mixed forests (Whitcomb et al., 1994, Dietrich, 1999, Stiller, 2009, Hamilton and Whitcomb, 2010). They also occur in temporary ruderal habitats such as meadows, pastures and farmland (Arenö, 1999, Nickel, 2003, Nickel and Achtziger, 2005). These habitats are usually characterized with large abundancy and diversity of leafhopper species (Hamilton and Whitcomb, 2010). In cereals fields and ruderal habitats *P. alienus* is one of the most abundant leafhopper species (Brcak, 1979, Nickel, 2003). The migration pattern of *P. alienus* is not well known. However, long-winged insects such as *P. alienus* may frequently move between grass populations and migrate to remote areas through passive wind dispersal (Raatikainen and Vasarainen, 1973, Nickel, 2003, Hamilton and

Whitcomb, 2010). Thus, factors such as wind speed, air temperature and wing length will have an important impact on the long-distance migration of leafhoppers (Waloff, 1980, Nickel, 2003).

1.7 Host plant – leafhopper – virus interaction

1.7.1 Partly overlapping distribution

WDV and *P. alienus* have been detected in regions of Asia, including the Fertile Crescent, which are partly overlapping with the range of wheat and its wild relatives. So far the occurrences of both the virus and the leafhopper have been reported from China and Syria (Xie et al., 2007, Zhang et al., 2010, Ekzayez et al., 2015). WDV has also been detected in Iran and Turkey (Köklü, 2004a, Köklü, 2004b, Behjatnia et al., 2011). However, relatively few surveys on occurrence of leafhoppers and WDV have been carried out in Asia, and their range may be larger than reported so far since the several host plants of WDV and *P. alienus* have a wide distribution within this region.

The overlapping distribution of the wheat relatives, *P. alienus* and WDV is influenced by the range of each species and the outcome of the interaction between them (Power, 2000, Hamilton and Whitcomb, 2010). Briefly described, the range of WDV is determined by the movement of the leafhopper vector and the ability to infect the host plants, and the leafhopper on its host preferences and the distribution of the host plants. The plant response and fitness, and thereby the range of the host plants are in turn partly affected by the virulence of the virus.

1.7.2 Coevolutionary relationship

In the regions where the distribution of the wild relatives of wheat, the leafhopper vector and WDV overlap a coevolutionary relationship may evolve (Hochberg et al., 2000, Occhipinti, 2013). The coevolution is based on reciprocal selection between the interacting species (e.g. Woolhouse et al., 2002). This can be seen as the defense of the infected plant towards the virus, counter-defense by the virus and the counter-counter-defense by the plant, forming an arms race between these species. Moreover, the change in feeding behavior of the leafhopper and its efficiency of transferring the virus may influence the coevolutionary relationship. An intensified selection on the host plant by the vector may result in development of traits making the plant less attractive to sap-feeding insects. This may in turn increase the intensity of selection on the vector and trigger a counter-response.

During the coevolution process the outcome of an interaction may range from being antagonistic to commensalistic, and mutualistic (Thompson and

Fernandez, 2006, Laine, 2009, Roossinck, 2011, Hily et al., 2015 and references therein, Roossinck, 2015). In an antagonistic relationship one of the species strongly benefits from the interaction, while a mutualistic relationship is beneficial to all of the interacting species. In commensalism one of the species benefits from the other, while the other species is not affected. Antagonism is common in cultivated fields, where the pathogens are causing severe damages on the susceptible crop plants (Brown and Tellier, 2011, Alexander et al., 2014). The cultivation of monocultures and cultivation practices, which does not allow any gene flow into the crop field, will not enable reciprocal selection between the pathogen and the cultivar and no coevolutionary relationship will evolve. Instead the evolution of resistance or tolerance to pathogens in crop plants will be dependent on breeding and human selection. Pathogens are also common in wild plant populations (Roossinck and García-Arenal, 2015 and references therein). They may, however, cause a variation in response within and among populations, where some plants are highly susceptible and others tolerant or resistant to the pathogen (Laine, 2009 and references therein, section Plant Responses).

1.7.3 Geographic mosaic theory of coevolution

The genetic variation within the interacting species is fundamental for their coevolutionary relationship and may in turn lead to different strength and direction of selection. The outcomes of the coevolutionary relationships are therefore a result of the interaction between the genotypes of the interacting species and the local environment, involving both abiotic and biotic factors. The interplay between these parameters are in general defined as the genotype-by-genotype-by environment (G x G x E) interaction. Change in any of these parameters may influence the coevolutionary relationship and the outcome may vary across geographical sites and over time. For example, variation in the abiotic factors influencing the local environment has been suggested to play an important role in several plant-pathogen interactions (Price et al., 2004, Barrett et al., 2007, Laine, 2008, Hily et al., 2015) Different stages of coevolution are therefore seen within and among populations.

Thompson (2005, 2013) integrated these aspects in his geographic mosaic theory of coevolution. He stated that spatial and temporal heterogeneity in strength and direction of reciprocal selection between two or more interacting species causes a geographic mosaic of hot spots, with intense reciprocal selection, and cold spots with no or weak selection acting on only one species. One would therefore expect hot spots of coevolution in regions with overlapping distribution of the interacting species, enabling strong reciprocal selection. Cold spots, on the other hand, are formed in sites, with non-

overlapping distributions of the species and thus preventing coevolution. Coevolutionary cold spots may even occur in sites with overlapping distribution, where commensalistic interactions have developed. In these interactions only the species benefiting from the interaction, will be under selection, and no evolutionary arms race will develop between the species. The intensity and direction of reciprocal selection may not only differ among sites and environments, but also change over time, and the evolutionary hot and cold spots may therefore shift within the geographic mosaic (Smith et al., 2011a). The shift in the coevolutionary dynamics may be caused by gene flow among plant populations, mixing adaptive traits evolved in different environments. It can also be affected by other population genetic processes such as differential random genetic drift among populations, mutations and extinction and recolonization of populations (Thompson, 2013).

2 Aims and hypotheses

Today's cultivars are the result of crossings, selections and in some cases gene modifications in long-term breeding programs for adaptation to present conditions. However, future crop plants are facing new challenges and multifaceted demands. Ideally, yields and crop nutritional quality should be higher, while using fewer inputs in crop production systems. On top of these demands the future crop plants have to be adapted to changes in climate conditions, and new pests and pathogens as a result of global warming. The general aim of the PhD study is to contribute to the development of improved cereals to pathogen resistance and tolerance, and in particular, to the development of virus resistant and tolerant wheat cultivars. As breeders are dependent on genetic resources for introducing new genes and traits in domesticated crops the search for genetic and phenotypic diversity relevant for the trait(s) of interest is crucial. The adaptation of wild crop relatives to different environments and biotic stresses make them potential genetic resources for crop improvement.

The large genetic diversity in the wild relatives of wheat and the partly overlap of distribution of these species and WDV, present an excellent opportunity to investigate the responses to virus infection in host plants of different genetic and evolutionary origin growing in various environments across the species ranges. Based on Thompsons theory of the geographic mosaic of coevolution (2005, 2013), where the intensity and direction of selection may vary over time and space a number of different taxa and genotypes directly or indirectly involved in the evolution of wheat and from different geographical sites were selected for the study. The different genotypes studied are from now on named accessions. These accessions were evaluated using an improved approach involving the plant-vector-virus system of wheat, the leafhopper vector *P. alienus*, and WDV.

Inspired by the geographic mosaic theory of coevolution, following hypotheses were tested:

- There is genetic variation in response to WDV among wild wheat relatives native to the Fertile Crescent and adjacent regions.

- There is genetic variation in response to WDV within *Ae. tauschii*, one of the wild ancestors of wheat and potentially important as a genetic resource.

- Growing environment of host plants generates variation in WDV response across the range of *Ae. tauschii*.

- Variation in WDV response is related to the evolutionary and genetic origins of accessions of *Ae. tauschii*.

- Domestication and human selection, and other genetic bottlenecks during wheat evolution such as natural hybridization and polyploidization events have had a negative impact on the resistance to WDV.

- The highly susceptible wild A genome donor *T. urartu* and the tolerant wild D genome donor of wheat *Ae. tauschii* differ in the onset of virus accumulation.

- Potential genetic resources as donors of genes and traits for development of WDV resistant and tolerant wheat cultivars will be identified among the wild relatives.

3 Materials and methods

3.1 Plant material

Domesticated and wild wheat taxa were selected based on their direct or indirect role in evolution history of hexaploid wheats (Petersen et al., 2006, Gustafson et al., 2009, Peng et al., 2011, Zohary et al., 2012). The wild accessions included nine *Aegilops* and three *Triticum* species, and *Amblyopyrum muticum* showing different ploidy levels and genome types. Among these species were the A genome donor of hexaploid wheat *T. urartu*, the putative B genome donor *Ae. speltoides* and the D genome donor *Ae. tauschii*. The wild accessions were collected within their native range in the Fertile Crescent and adjacent areas in West Asia. Moreover, 33 accessions of *Ae. tauschii* in four different biomes, the Temperate grasslands, savannas and shrublands, Montane grasslands and shrublands, Temperate broadleaf and mixed forest, and Desert and xeric shrublands across the species range in West Asia were further studied (Jones et al., 2013). These accessions belong to two different evolutionary lineages and are genetically structured in six sub-populations.

The domesticated wheat taxa included the diploid cultivated einkorn, the tetraploids cultivated emmer and durum, and the hexaploids spelta and bread wheat. Two winter cultivars of bread wheat, Tarso and Kosack, were used. Seeds of these cultivars were provided by the breeding company Lantmännen SW Seed AB, Svalöv, Sweden. Seeds of the other accessions were provided by the International Center for Agricultural Research in the Dry Areas, previously in Aleppo, Syria, and the John Innes Centre, Norwich, UK.

3.2 Regenerating host-vector-virus system

A regenerating host-vector-virus system was successfully developed to provide WDV carrying leafhoppers for transmission of the virus to the studied host

plants. Transmission of WDV by viruliferous leafhoppers is the only reliable and efficient method for virus transfer to plants (Vacke, 1964, Woolston et al., 1988, Ramsell et al., 2005, Ramsell et al., 2009), and in our studies a near 100% transmission efficiency was found. *P. alienus* leafhoppers were collected in different wheat fields in central Sweden (Figure 1). These leafhoppers were reared in nylon mesh covered cages (17 cm x 13 cm x 13 cm) on wheat source plants kept in a greenhouse with 16/8 hrs day/night photoperiod and 20/18 ºC day/night temperature (Figure 5). In the same wheat fields the first generation of source plants was collected. These plants were confirmed to be WDV infected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). In the regenerating host-vector-virus system old infected source plants where regularly replaced by new healthy wheat plants, which during exposure to viruliferous leafhoppers, became infected. In turn, newly hatched and virus-free nymphs acquired WDV from infected wheat plants while feeding on them.

Figure 5. Wild wheat plants in cages with viruliferous *Psammotettox alienus* leafhoppers.

3.3 Greenhouse experimental designs

In Papers I and II the experimental host plants were grown in a complete randomized block design with six and four blocks, respectively. Each block consisted of two plants of each of the studied accessions. In Paper I all the blocks were running in parallel. In Paper II twice as many accessions were evaluated and in order to minimize the time used to evaluate the plants at each time point and developmental stage the blocks were repeated at different time points. Each plant was cultivated in a separate 2L pot and placed into a cage with nylon fabric with fine mesh (17 cm x 13 cm x 13 cm) to keep the leafhoppers inside the cage. At the 2nd leaf stage one of the two plants of each accession and block was exposed to three viruliferous leafhoppers, both adults

and nymphs from the regenerating host-vector-virus system described above, for seven days, whereas the other plant was not exposed. The randomization of accessions and treatments in each of the blocks enabled us to estimate the block effect and also to reduce the environmental effect on the variation of the studied phenotypes among accessions.

In Paper III, however, the randomization of plants in blocks would have made it more complicated to carry out the study. In this study plants were analyzed for virus content at different developmental stages (1st through 5th leaf stage) and a surplus of plants of each accession (about 40 plants) was grouped together in the greenhouse to facilitate the selection of plants at the appropriate leaf stage. The accessions were placed next to each other on the same table. Single plants at the 1st leaf stage were placed in cages described above and exposed to three viruliferous leafhoppers for three days.

3.4 Evaluation of response

3.4.1 WDV content

The accumulation of virus in leaves and roots of exposed and non-exposed plants was analysed with DAS-ELISA. This is the standard method for detection and quantification of virus content in large number of samples, especially in agricultural crops (Šíp et al., 2006). Samples with very low absorbance values when analysed by DAS-ELISA, were further analysed by immunocapture-polymerase chain reaction (IC-PCR). In addition, some plants with low absorbance value were re-extracted and tested with polymerase chain reaction (PCR). The protocol used for analyses with DAS-ELISA is given in Paper I, the protocol for IC-PCR in Paper II, and the protocol for PCR in Paper III.

3.4.2 Response traits

A number of different traits were chosen for the study of response patterns. As leaf chlorosis and reduced growth are commonly associated with WDV infection in wheat fields, leaf chlorosis (ratio of number of chlorotic leaves to total number of leaves - a leaf was regarded as chlorotic when at least 50% of the leaf area was yellow), plant height, shoot dry weight, and number of leaves and tillers were selected for the investigation of variation in response in wild and domesticated wheat. These traits were evaluated at different time-points to increase our understanding of the response pattern over time. To our knowledge this is the first study which has investigated the response to WDV infection in wild and domesticated wheat by combining the analyses of leaf

chlorosis, different growth traits and WDV content at different time points and plant developmental stages.

As the study comprised accessions with different genetic and environmental origin, variation in the constitutive developmental patterns and morphology was expected. Thus, to be able to compare accessions within and between time points we have evaluated the response as the absolute difference between the non-exposed and exposed plants for the same accessions, and as the proportional difference (ratio of absolute difference and measurement in the non-exposed control condition) between plants in the two treatments. Because of differences in the experimental design between the trials explained above, the equation of the absolute and proportional difference is accordingly adapted and presented in each of the papers (I, II, and III).

3.4.3 Data analysis

The data were analyzed using the parametric test analysis of variance (ANOVA) to evaluate if the residuals for each trait followed normal distributions. For traits showing normally distributed residuals the ANOVA test could be applied. However, when non-normally distributed residuals were found for a trait and the residuals could not be stabilized by transformation the data were analyzed with the non-parametric Kruskal–Wallis test. To analyze and display relations among patterns of responses in wild wheat accessions based on data from all response traits we used principal component analysis (PCA).

4 Results and discussion

4.1 Large variation

As a first step to increase the understanding of the variation in response to WDV and to identify potential genetic resources, we selected different wild species with different genome type and ploidy level, and from different geographic locations rather than a larger number of accessions within a few species. The large diversity has most likely increased the chances of variation in intensity of selection and strength of coevolution between the virus and the host plants, and thereby increased the ability to identify genetic resources for wheat breeding.

Among thirteen species of the wild wheat relatives a large variation in response was found (Paper I). These wild relatives showed moderate to high levels of WDV at 28 days after the end of exposure (dpi) to viruliferous leafhoppers. At the same time point all of them showed a higher percentage of leaf chlorosis and a lower number of tillers and leaves (except wild emmer) in the infected plants compared to the non-infected plants, even though a milder response was seen in some wild accessions (significant accession x treatment interactions; $P<0.05$, two-way ANOVA). When comparing the plant response at 28 dpi with the response at a later time point (98 dpi for tiller number, and 112 dpi for leaf number and shoot dry weight) different response patterns were found. Interestingly, some *Aegilops* species such as the D genome donor *Ae. tauschii* showed a decrease in the proportional reduction of tiller and leaf number between non-exposed and exposed plants over time (Figure 5, Paper I). On the contrary, other *Aegilops* species such as the putative B genome donor of wheat *Ae. speltoides* showed a continued increase in the proportional reduction for the same traits. Moreover, the mortality in infected plants of the A genome donor of wheat *T. urartu*, and wild einkorn, the ancestor of cultivated einkorn was high. The variation in responses can be summarized in three different

patterns: *i)* continuous reduction in growth over time, *ii)* weak response at an early stage of plant development but a much stronger response at a later stage, and *iii)* remission of symptoms over time. However, variation in response was found within these categories and they should not be seen as distinct patterns.

4.2 Large variation in response within *Aegilops tauschii*

The third category of plant response with remission of symptoms over time may indicate some degree of tolerance against WDV. We therefore found it interesting to further investigate host plants showing this response pattern as a next step towards achieving our goal. *Ae. tauschii* is one of the accessions which showed a remission of symptoms with increased growth and lower percentage of leaf chlorosis over time. This is particularly interesting as its D genome shows high similarity with the D genome in hexaploid bread wheat and has been used as the diploid parent in crosses with tetraploid wheat (*T. turgidum*) to produce synthetic hexaploid wheat (Li et al., 2014 and references therein). By studying different accessions of *Ae. tauschii* we got the opportunity to further explore its potential as genetic resource and to test whether the three response patterns found among wild relatives also applies at the within-species level (Paper II).

The WDV content was significantly different among accessions at the two time points (28 and 56 dpi; $P<0.01$, Kruskal-Wallis test) and the change in WDV content between the time points varied among accessions ($P<0.001$, Wilcoxon test, Figure 2a, Paper II). Interestingly, five of the *Ae. tauschii* accessions had very low WDV content at both time points. The infected plants of these accessions showed similar absorbance values with DAS-ELISA as the non-infected plants. Even though the WDV content was low, these plants were confirmed to be infected by IC-PCR. In the other accessions the WDV content varied from moderate to high levels at 28 dpi. However, at 56 dpi most of these accessions showed a significant decrease in WDV content compared to the first time point ($P<0.001$, Wilcoxon test), particularly, in two accessions, where the WDV content was reduced to similar level as the non-infected plants.

Different response patterns were also found for leaf chlorosis. A few *Ae. tauschii* accessions showed a mild response at both time points, whereas the leaf chlorosis increased significantly over time for some accessions ($P<0.01$, Wilcoxon test, Figure 2b, Paper II). Most of the accessions, however, showed a remission of symptoms as smaller difference in leaf chlorosis between the non-infected and infected plants was found at 56 dpi than at 28 dpi. Notable is that the infected plants of seven of them had lower percentage of chlorotic leaves

than the non-infected plants of the same accessions. Three of them were among the accessions which had very low WDV content at both time points.

There was also a significant difference in response in leaf number over time among accessions ($P<0.001$, three-way ANOVA). A few accessions showed almost no effect of the WDV infection at 28 dpi (Figure 2c, Paper II). They had, however, a later onset of symptoms and showed a major deterioration in growth with nearly 80% reduction in leaf number compared to the non-infected plants at 56 dpi. Three of the accessions with a low WDV content at both time points showed mild symptoms throughout the growth. Among the accessions which had an early onset of symptoms, three-fourths showed a further reduction in leaf number compared to the non-infected plants at 56 dpi. However, for many of them the reduction in growth diminished over time. The remaining fourth with an early onset of symptoms showed a decrease in symptoms between 28 and 56 dpi. A further decrease in symptoms during the growth of the plant, studied at 98 and 112 dpi, was seen in some accessions in our study on different wild relatives (Paper I).

For the purpose of obtaining a more complete picture of the variation in response, the data of WDV content and the different response traits at different time points were analyzed with the multivariate method PCA (Paper II). A large variation was found for the *Ae. tauschii* accessions when the combined response of many traits were analyzed. Based on the PCA and the single trait analyses *Aegilops* showed the same response patterns as described for the wild relatives. In addition, partial resistance was found in some accessions of *Ae. tauschii*.

4.3 Tolerance and partial resistance in species of *Aegilops*

Comparing the results from the studies in Paper I and II the three different response patterns found among wild wheat relatives were also found within *Ae. tauschii* (Figure 6) The two response patterns with a continuous increase of symptoms during growth, and an initial weak but strong response at a later developmental stage show susceptibility to WDV infections. In contrast, some wild accessions such as *Ae. cylindrica*, *Ae. searsii* and *Ae. tauschii* were able to constrain the development of symptoms (Paper I and II). Even though the spread and amplification of WDV was not completely restricted in any of the *Ae. tauschii* accessions, two different response patterns to limit the damage caused by the virus seem to have evolved in some of the accessions (Paper II). A few accessions showed low WDV content at both time points and weak symptoms throughout their growth, which suggests some level of partial resistance. Moreover, most of the accessions with an initially high WDV

content showed a large reduction in virus content over time. Some of these accessions had a remission of symptoms at later developmental stages. This remission of symptoms was also seen in *Ae. cylindrica* and *Ae. searsii* (Paper I). This ability to recover from the early onset of symptoms could be considered as WDV tolerance.

The remission of symptoms seen in *Aegilops* is may be associated with a reduction of virus content triggered by the RNA silencing system (Ghoshal and Sanfaçon, 2015). This plant response restricts the accumulation and spread of viruses within the plant. However, as a counter-defense the viruses have evolved RNA silencing suppressor proteins (RSS). These RSSs are also interfering with cellular processes regulated by RNA silencing and are thereby affecting the growth and development of the host plant (Chapman et al., 2004, Pallas and García, 2011, Smith et al., 2011b). The disturbance in growth and development triggers a reprogramming of host plant responses, which in tolerant plants will result in a reduction of virus amplification and movement (Bengyella et al., 2015). Differences in transcriptome reprogramming have been found in germinivirus infected cassava, where changes in gene expression have been found between susceptible and tolerant plants (Allie et al., 2014).

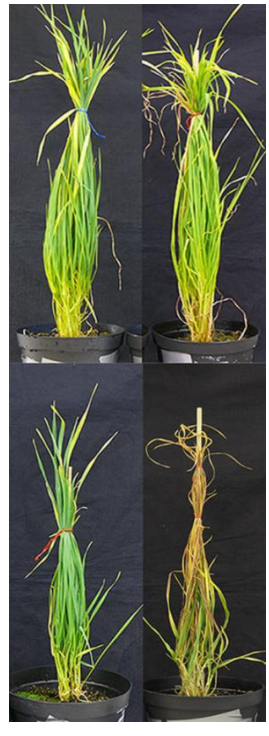

Figure 6. Large variation in response to WDV infection in *Ae. tauschii*. Top: *Left* Nonexposed *Ae. tauschii* plant, *right* partially resistant; Bottom: *Left* tolerant, *right* susceptible.

4.4 Variation in response is partly affected by environmental origin in *Ae. tauschii*

Ae. tauschii has a large native distribution, which range from the Caucasus region between the Black Sea and the Caspian Sea in the west to China in the east. It grows at different altitudes and in locations which vary greatly in temperature, precipitation and humidity, as well as plant species composition (Jones et al., 2013). The occurrence in different habitats and the large variation in response to WDV found within the species gave us the opportunity to test whether the environmental origin of the host plants influences the response to WDV (Paper II). The *Ae. tauschii* accessions we evaluated in our study were assigned to four different biomes, the Temperate grasslands, savannas and shrublands, Montane grasslands and shrublands, Temperate broadleaf and mixed forest, and Desert and xeric shrublands (Jones et al., 2013). Each biome was dispersed throughout the species range and occurred in different geographical locations.

The exposed accessions in the four biomes differed significantly from each other in WDV content at 28 dpi ($P<0.05$, Kruskal-Wallis test), but not at 56 dpi. The lowest mean WDV content was found in the plants from the Temperate grasslands, savannas and shrublands at both time points and three of the accessions with very low WDV content were collected in this biome. The other two accessions with low WDV content were growing in the Montane grasslands and shrublands and the Desert and xeric shrublands, respectively. Accessions from the Montane grasslands and shrublands and the Temperate broadleaf and mixed forest had the highest mean WDV content at both time points. The accessions differed also significantly in leaf number and shoot dry weight among the biomes at 56 dpi ($P<0.05$, two-way ANOVA). A significant difference was also found over time among the biomes ($P<0.001$, three-way ANOVA). The plants in the Temperate grasslands were least affected for the two traits, whereas the accessions in the Montane grasslands and shrublands and the Temperate broadleaf and mixed forest showed on average the most severe symptoms. However, no significant difference was found in leaf chlorosis among biomes.

Using the multivariate method PCA on the response traits, the variation in response was partly structured according to biomes (Paper II). Most of the *Ae. tauschii* accessions in the Temperate grasslands, savannas and shrublands grouped together in the score plot. Based on the results of the multivariate, ANOVA and Kruskal-Wallis analyses, the environmental origin has an effect on the plant response to WDV. Less susceptible plants with lower WDV content and milder symptoms were found in the Temperate grasslands, savannas and shrublands. This suggests that the studied accessions in this

biome are on average better adapted to WDV than accessions in the other biomes. Based on the geographic mosaic theory of coevolution (Thompson, 2005) this could be explained by strong reciprocal selection between the host plant and WDV. The strength of selection in the coevolutionary relationship between *Ae. tauschii, P. alienus* and WDV may be stronger in habitats with large dense grass populations such as the Temperate grasslands, savannas and shrublands biome than in more herogenous habitats with sparse, patchy grass vegetation and large diversity of non-host plants families. Larger areas of grasses has been found to have a higher immigration rate and density, and a lower extinction rate of planthoppers, species within the same suborder (Homopera) as *P. alienus*, than smaller areas (Cronin, 2003). The presence of the WDV vector and the virus will insert a strong selection on the host plant and a counter-defense will result in an evolutionary arms race between the interacting species. This arms race may result in two different evolutionary outcomes. The population may reach equilibrium with a mix of resistant and susceptible host plants in the population. This is caused by negative-frequency dependent selection, where the resistance is favoured by the selection as long as there is a fitness advantage of being resistant. The advantage will, however, decline when the frequency of the resistant plants increases in the population and the chance of becoming infected decreases (e.g. Roy and Kirchner, 2000). If the susceptible host plant instead evolves tolerance (reducing the fitness consequences of the host plant without reducing the fitness of the virus) the incidence of infections will increase and the advantage of being tolerant increases. This may lead to fixation of tolerance genes in the population. However, if resistance or tolerance is coupled to other traits which impose a cost on the fitness of the non-infected plant such as reduced growth and reproduction, the spread of resistance and tolerance genes within a population will be hampered (e.g. Huot et al., 2014). Trade-offs between defense and vegetative growth and reproduction in host plants are caused by changes in the allocation of restricted resources. This process is suggested to be mediated by interacting defense and growth signalling pathways.

The other biomes showed a larger variation in response than the Temperate grasslands, savannas and shrublands, and tolerant accessions were also identified in the Montane grasslands and shrublands, the Temperate broadleaf and mixed forest, and the Desert and xeric shrublands. These biomes may be more heterogenous than the Temperate grasslands, savannas and shrublands and thereby present a larger within-biome variation in strength of selection. The variation in intensity of selection within and between biomes is one of the factors generating the variation in response within *Ae. tauschii*.

4.5 Response pattern is not related to the evolutionary and genetic origin of *Aegilops tauschii*

Analyses based on genetic markers revealed two distinct evolutionary lineages of *Ae. tauschii* (Lubbers et al., 1991, Dvorak et al., 1998, Mizuno et al., 2010, Sohail et al., 2012, Jones et al., 2013, Wang et al., 2013). These two lineages were also represented among the accessions studied. These accessions are further structured in six subpopulations, of which four belong to the lineage which is most closely related to bread wheat, and the other two subpopulations to the other lineage (Figure 1, Paper II, Jones et al., 2013). The geographic locations of the six subpopulations do not correspond with the locations of the four biomes, so that accessions from the same biome are from different subpopulations (Figure 1, Paper II). The known population genetic structure of *Ae. tauschii* accessions gave us the opportunity to investigate the effect of the genetic origin on the variation in response to WDV.

Compared to the biome origin, the genetic origin had minor effect on the variation in response to WDV. The subpopulations did not differ significantly in WDV content and in shoot dry weight, but showed significant difference in leaf number over time ($P<0.01$, three-way ANOVA) and leaf chlorosis at 56 dpi ($P<0.05$, Kruskal-Wallis test). However, using the multivariate analysis PCA for studying the combined effect of symptomatic traits we did not find any evidence for grouping of accessions based on subpopulations (Paper II). Knowing that the biome of the *Ae. tauschii* accession has an effect on the response pattern to WDV, and the geographic locations of the subpopulations and biomes are incongruent, we did, however, not expect to find a strong correlation between the variation in response and the genetic origin of *Ae. tauschii* accessions.

4.6 Antagonistic and commensalistic relationships between wild wheat relatives and WDV

The interaction between wild wheat relatives and WDV has resulted in different evolutionary outcomes. High susceptibility in host plants, for example *T. urartu*, the A genome donor of wheat, may be a result of weak selection in evolutionary cold spots, or seen in interactions at an early phase of the coevolving dynamics (Laine, 2009). Susceptible plants may also remain in populations with resistant plants through negative-frequency dependent selection as described above. Partial resistance and tolerance in accessions of *Aegilops* are other outcomes of reciprocal selection within the host-vector-virus interaction. However, compared to complete resistance, partial resistance could weaken the evolutionary arms race as the virus is maintained at low levels in

the host plant. An interaction where the host plants show high susceptibility and only the virus benefits from the interaction may be seen as an antagonistic relationship. Tolerant plants reduce the consequences of the WDV infection without reducing the fitness of the virus. This strategy may be more evolutionary stable than antagonism and may be seen as commensalism, where WDV benefits from the relationship without harming the host plant (Roy and Kirchner, 2000). The relaxed arms race may lead to closer associations between the virus and the host plant and thereby facilitate the shift from commensalism to mutualism. However, the evolution of this interaction is strongly influenced by the leafhopper vector, for example through its efficiency in transferring the virus and its movement within and between host plant populations, which is partly dependent on its preferences of host plants. The host preference can be influenced by the pathogen itself as some viruses have been found to manipulate the host plant to attract insect vectors for feeding (e.g. Ingwell et al., 2012, Mauck et al., 2012, Hu et al., 2013, Liu et al., 2013, Rajabaskar et al., 2014). In addition, other organisms and abiotic factors will influence the coevolutionary relationship. The community as well as the habitat fragmentation should therefore play a role in the evolutionary outcome of the interaction, which our findings also confirm (Paper II).

4.7 No general impact of domestication on the response to WDV

To further increase the understanding of the underlying causes to the variation in response to WDV we investigated whether the evolutionary genetic bottlenecks caused by domestication and selective breeding have resulted in a loss of tolerance and resistance in cultivated wheats (Paper I). Wild wheat relatives and diploid, tetraploid and hexaploid wheats involved in the evolution of bread wheat were evaluated for response to WDV. The wild and domesticated accessions were grouped separately and the two groups were compared for their response to WDV. Even though the domesticated group showed significantly higher WDV content ($P<0.05$, t-test) the two groups did not differ in symptomatic traits (leaf chlorosis, number of leaves and tillers and shoot dry weight). These results suggest that the loss of genetic diversity expected by the domestication process and other bottlenecks such as natural hybridization and polyploidization events during wheat evolution have not resulted in a general increase in susceptibility to WDV infection. The reduced genetic diversity caused by these bottlenecks may have been compensated by the hybridization of the different ancestral genomes followed by duplication, resulting in new genetic diversity. In addition, the polyploid nature of

tetraploid and hexaploid wheats enables buffering capacity and greater robustness against gene mutations.

Like in the wild relatives the domesticated wheats showed large variation in response to WDV and the three different response patterns found in the wilds were also represented in the domesticated wheats. Durum wheat and cultivated einkorn had clear symptoms already at the first time point at 28 dpi, and showed a continuous reduction in growth at the second time point (98 dpi or 112 dpi). Interestingly, an opposite response was found in spelt wheat, which showed mild symptoms at 28 dpi and an increase in growth over time. In fact, the growth between the first and the second time point was even higher in the exposed than in the non-exposed plants. It has been suggested that increased vegetative production in BYDV infected oat is caused by reallocation of host plant resources resulting in vegetative growth rather than spike production (Persson et al., 2007). We did, however, not find any evidence for that in our study. Notable is that the exposed plants of spelt had the highest WDV content at 28 dpi of all the studied accessions, and the average absorbance value tested by DAS-ELISA was more than twice as high compared to the values for several wild accessions. The mild response in spelt despites its high content of WDV, suggests that this accession is tolerant to the virus.

Like spelt wheat, cultivated emmer and the bread wheat winter cultivar Tarso had a mild response at the first time point. They showed, however, much stronger response at time of harvest than at 28 dpi. The response was particularly severe in cultivated emmer and all plants of this accession died before the end of the experiment. Tarso as well as another winter cultivar Kosack were evaluated together with the *Ae. tauschii* accessions in Paper II. The continued reduction in growth over time found for Tarso in the study including both wild and domesticated wheat (Paper I) was confirmed in the second study on *Ae. tauschii* (Paper II), even though Tarso was highly affected already at the first time point in the latter study. However, the effect on leaf chlorosis during growth (which was only studied at 28 dpi in Paper I) was less severe and decreased. The WDV content was higher in Tarso than in most of the accessions of *Ae. tauschii* and several other *Aegilops* species. The winter cultivar Kosack, only included in the second study, had more severe symptoms than Tarso, particularly in leaf chlorosis, and most *Ae. tauschii* accessions were considerably less affected than Kosack at both time points (28 and 56 dpi). Kosack had also higher WDV content than Tarso and most of the *Ae. tauschii* accessions. Variation in response among bread wheat cultivars has also been found in other studies (Vacke and Cibulka, 2000, Vacke and Cibulka, 2001, Lindblad and Waern, 2002, Benkovics et al., 2010).

4.8 Later onset of virus accumulation in *Aegilops tauschii* than in *Triticum urartu* and bread wheat

The lack of correlation between WDV content and severity of symptoms in the different *Aegilops* and *Triticum* species which we have studied, may at first be surprising as one may expect that the rate and extent of the virus amplification and spread within the host plant will influence the severity of symptoms. However, the severity of symptoms may rather be linked to the activity of the RSSs affecting the growth and development by interfering with cellular processes in the host plant, than the virus content (Chapman et al., 2004, Pallas and García, 2011, Smith et al., 2011b). On the other hand, restriction of virus replication and spread is a common form of plant defense (Niehl and Heinlein, 2011, Vuorinen et al., 2011, Hipper et al., 2013), and symptom recovery is often coupled with reduced virus content (Ma et al., 2014, Nie and Molen, 2015). In Paper I and II the correlation between WDV content and plant response was studied at later stages of plant growth. Thus, to further explore the effect of WDV on the different response patterns in wild wheat accessions (Paper I and II), we have investigated the WDV content in leaves and roots of host plants at different stages early in their development (first through fifth leaf stage, Paper III). This was done by DAS-ELISA and in the samples with low DAS-ELISA absorbance values the presence or absence of WDV was studied by PCR. Based on the variation in response pattern among wild and domesticated wheats studied in Paper I, we selected the highly susceptible accession of *T. urartu*, the less susceptible winter cultivar Tarso and the tolerant *Ae. tauschii* accession for comparison (Figure 7).

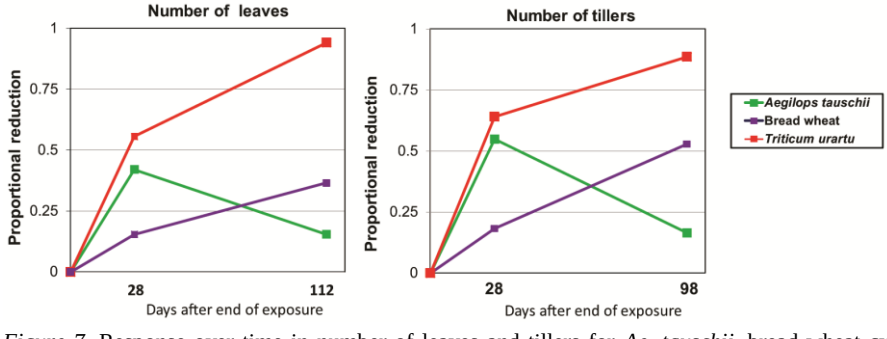

Figure 7. Response over time in number of leaves and tillers for *Ae. tauschii*, bread wheat cv. Tarso and *T. urartu*.

The virus was detected in the roots already at the first leaf stage of all accessions and increased in the roots throughout the early development of the plants. The virus was later detected in the leaves, but the onset of virus spread

from the first leaf, inoculated with WDV, to the younger leaves and the level of accumulation varied among the studied accessions. The virus reaches the younger leaves through a cell-to-cell movement within mesophyll cells in the first leaf, and when it has reached the phloem it is passively transported within the source-to-sink flow of photoassimilates (e.g. Hipper et al., 2013).

T. urartu was the accession showing the first sign of WDV accumulation in the leaves and WDV was detected already at the second leaf stage. The WDV content was further increased in the second leaf at the third leaf stage. *T. urartu* had considerably higher mean WDV content at the third leaf stage than the highest WDV content found in cv. Tarso and *Ae. tauschii*. All plants of *T. urartu* were severely affected at the fourth leaf stage and they died before they reached the fifth leaf stage. Both the winter cultivar Tarso and *Ae. tauschii* showed presence of WDV in leaves at the fourth leaf stage. However, while WDV was found in the second, third and fourth leaf in Tarso, only the two youngest leaves showed presence of WDV in *Ae. tauschii*. Moreover, the virus content was considerably lower in the two youngest leaves of *Ae. tauschii* than in Tarso. At the fifth leaf stage all leaves in both accessions showed presence of WDV. These findings suggest a later onset of accumulation in the shoots and a delayed systemic infection in the tolerant *Ae. tauschii* compared to both *T. uratu* and Tarso. *T. uratu* showed the highest WDV content and the fastest spread of the virus within the plant, which resulted in premature dead. Compared to the other accessions, a significantly higher percentage of leaf chlorosis and lower leaf and root fresh weights were found in *T. urartu* at earlier leaf stages. The severe symptoms on *T. urartu* suggest that the plant developmental stage at the time of onset of virus spread and the level of virus accumulation have a significant effect on the plant response to WDV early in plant development. The delayed onset of virus accumulation and thereby later onset of defense mechanisms during plant development may be one of the factors which have made it possible for *Ae tauschii* to tolerate the systematic infection and to continue growing.

4.9 Potential genetic resources in wild *Aegilops* relatives and a wheat landrace carrying the D genome

Our results clearly show a variation in response pattern to WDV among wild and domesticated wheats. This variation ranges from highly susceptible to partially resistant and tolerant plants. Tolerance to WDV, where the plant shows continued growth and remission of symptoms over time, was found in four wild *Aegilops* species and in spelt wheat, one of the domesticated wheat taxa. They show different ploidy level, but, interestingly, spelt wheat and three

of the wild species, *Ae. juvenalis*, *Ae. cylindrica* and *Ae. tauschii* are carrying the D genome. A few *Ae. tauschii* accessions showed also mild symptoms and some degree of restriction of virus accumulation throughout the plant growth, suggesting that these accessions are partially resistant to WDV. Even though our results may not be broadly applied to a species level since only one accession of each species have been studied, except for *Ae. tauschii*, our findings indicate that the response pattern to WDV is associated with the genome type.

None of the studied accessions showed, however, complete resistance (immunity), where the colonization of the virus is fully restricted. Complete resistance may more easily evolve against specific groups of the pathogen, which are genetically similar (race, isolate). As wild plants are exposed to a variety of pathogens and genotypes, complete resistance may therefore not be an efficient defense strategy in natural populations. It has a simple Mendelian inheritance and this qualitative resistance is easily overcome by the pathogen in the evolutionary arms race (Mehta, 2014). However, partially resistance is a quantitative trait controlled by many genes of different magnitude of effect, which may be more difficult to overcome by the pathogen. This type of defense is considered to be non-genotype specific and of longer duration, and thereby of interest for disease resistance breeding (Mehta, 2014, Brown, 2015). However, according to our findings tolerance is the most frequent defense strategy in the wild relatives of wheat. It is most likely also governed by many genes, possibly different from the genes controlling partial resistance, and may be efficient and against different genotypes and pathogens. Considering the more stable interaction between a tolerant host plant and a pathogen due to relaxed reciprocal selection, breeding of tolerance provides an additional strategy for improvement of crops. The partial resistant and tolerant *Aegilops* accessions as well as the tolerant accession of spelt wheat are therefore potential genetic resources for breeding of resistance and tolerance to WDV in wheat. *Ae. tauschii* is of particular interest as this diploid can be hybridized with tetraploid durum or emmer wheat, carrying the A and B genomes, to form synthetic hexaploid wheat (Dreisigacker et al., 2008, Ogbonnaya et al., 2013, Li et al., 2014). Notable is that spelt wheat is considered as a landrace rather than a cultivar and may therefore show more genetic diversity than bread wheat cultivars which are the products of formal breeding programs. This landrace may therefore harbor further diversity of interest for tolerance and resistance to WDV. In view of our findings from the study of *Ae. tauschii* accessions, the diversity of response to WDV in other *Aegilops* species should be further explored.

5 Summary of findings

In view of the hypotheses tested using an improved approach involving the plant-vector-virus system of wild and domesticated wheat, the leafhopper vector *P. alienus*, and WDV the main findings are summarized as follows:

- Wild wheat relatives native to the Fertile Crescent and adjacent regions show large variation in response to WDV. This variation can be summarized in three different response patterns: *i)* continuous reduction in growth over time, *ii)* weak response at an early stage of plant development but a much stronger response at a later stage, and *iii)* remission of symptoms over time. The first two patterns include different levels of susceptibility, whereas the third pattern suggests a tolerance strategy.

- Similar response patterns were found within the wild relative *Ae. tauschii*, potentially important as a genetic resource. Moreover, a few *Ae. tauschii* accessions showed a fourth response pattern: *iv*) low WDV content and mild symptoms throughout the plant growth, indicating partial resistance.

- The variation found in *Ae. tauschii* is partly explained by its adaptation to different growth environments. Less susceptible plants with lower WDV content and milder symptoms were found in the biome Temperate grasslands, savannas and shrublands. The variation found both within and among biomes may be explained by the geographic mosaic theory of coevolution (Thompson, 2005), where differences in the intensity of reciprocal selection between the interacting species will create a mixture of evolutionary hot and cold spots across space and time.

- On the contrary, the variation in WDV response is not related to the evolutionary and genetic origin of the diploid wild wheat ancestor *Ae. tauschii*.

- Domestication and human selection, and other genetic bottlenecks during wheat evolution such as natural hybridization and polyploidization events have not resulted in a general increase in susceptibility to WDV in cultivated wheats. Instead, variation in response was found among the diploid, tetrapoid and hexaploid wheats.

- The highly susceptible wild A genome donor *T. urartu*, a less susceptible winter wheat cultivar and the tolerant wild D genome donor of wheat *Ae. tauschii* differ in the onset of virus spread and level of WDV accumulation at early stages of development. The tolerance in *Ae. tauschii* may partly be explained by the later onset of systemic infection.

- In an evolutionary context, the interaction between wild wheat relatives and WDV has resulted in different outcomes. Susceptibility in host plants may be found in an early phase of coevolution. This interaction is antagonistic as only the virus benefits from it. Tolerance, which is the most common defense strategy in wild wheat relatives, reduces the consequences of the virus without reducing its fitness. This strategy may be seen as commensalism.

- Potential genetic resources for improvement of tolerance and resistance to WDV are identified in wild *Aegilops* species and a landrace of spelt wheat. They have different ploidy levels, but almost all of them are carrying the D genome, one of the three genomes in hexaploid wheats. Of particular interest are the tolerant and partial resistant accessions of *Ae. tauschii*, the D genome donor or wheat. This diploid can be crossed with tetraploid durum and emmer wheat and form synthetic hexaploid wheat. These findings indicate that the response to WDV may be associated with the genome type.

6 Implications and future perspectives

6.1 A regenerating host-vector-virus system for future studies

In light of the knowledge and experience obtained from the studies on the interaction between wild wheat relatives, the leafhopper vector *P. alienus* and WDV conducted in this PhD thesis, this interaction presents an excellent system for empirical studies. This species interaction is likely to be used in studies of various perspectives including genetic and molecular mechanisms of plant defense, coevolutionary genetics and biology and pre-breeding of disease resistance and tolerance, and thereby involving studies from DNA and cellular levels to individual, population and community levels.

Experimental studies on species interactions are often challenging as two or more organisms need to be handled. Thus, the regenerating host plant-vector-virus system, which we successfully developed, has been crucial for carrying out the experiments presented in this PhD thesis. Even though these studies are challenging they are also very exciting as they will result in both improved fundamental and applied knowledge. By studying the response to WDV in wild relatives of wheat at different developmental stages we have deepened the understanding of the diversity of response patterns and the variation in the onset of the systematic infection. The variation found in response could be referred to different defense strategies and outcomes of the coevolutionary relationship. These findings can be applied in basic research on defense mechanisms as well as in further pre-breeding research aiming for development of adapted breeding programs and crop cultivation practices. Besides studying the interaction from the host plant perspective the pre-breeding research would gain from getting a more profound understanding of this interaction. In particular, the influence of the *P. alienus* vector on the interaction is not well known and it would be of interest to focus on the two-species interactions between the leafhopper and the virus, and between the

leafhopper and the plant, as the next step. Moreover, as the variation in response among accessions reflects different phases of coevolution this host plant-vector-virus interaction presents an excellent opportunity to test the geographic mosaic theory of coevolution.

6.2 Pre-breeding for effective breeding programs

Diseases caused by viruses and other pathogens in our cultivations are difficult to control by pesticides and cultivation practices alone. Another concern is the pollution of water and soil by the pesticides, causing negative effects on the environment, ecosystems and human health. Repeated use of pesticides may also evolve resistance to pesticides in the pathogens and insect vectors. Thus, improvement of resistance and tolerance in crop cultivars is the most effective and environment-friendly solution to reduce damage by pathogens. However, breeding is a long-term and costly process. Pre-breeding research, where this PhD thesis is a part of, is crucial for developing effective and well-designed breeding programs.

Farmers are dependent on cultivars which show durable resistance or tolerance to pathogens for high yield. The durability is influenced by the relatively ease with which the pathogen will evolve virulence and overcome the resistance. This process is in its turn affected by the number of genetic changes needed to acquire virulence and the degree of virulence achieved by these mutations (Harrison, 2002, Lecoq et al., 2004). Resistance controlled by single major genes, which are specific to certain genotypes (fungal races and virus strain) of the pathogen, are considered to be less durable than partial resistance. Resistance to power mildew in barley is, however, an example of a durable resistance controlled by a single gene (Piffanelli et al., 2004). Partial resistance is non-specific and can act against different genotypes and pathogens. It is controlled by many genes of various individual effects and may be more difficult to overcome by the pathogen (Poland et al., 2009, Kou and Wang, 2010). In addition, partial resistant plants keep the pathogen at low levels without completely restrict its colonization. This defense strategy weakens the intensity of selection on the pathogen and thereby the evolution of increased virulence. In contrast to barley, durable resistance to power mildew in wheat has been gained by partial resistance (Brown, 2015). The durability of this defense strategy makes therefore the partial resistant accessions of *Ae. tauschii* of great interest for breeding of resistance to WDV in bread wheat. Moreover, the larger number of *Ae. tauschii* accessions showing tolerance to WDV is also potential genetic resources. Like partial resistance, tolerance is most likely also controlled by many genes and may be efficient and against

different genotypes and pathogens. The interaction between tolerant *Ae. tauschii* accessions and WDV may be seen as a commensalistic relationship, where the virus gains from the relationship without severely harming the host plant. The commensalism and the polygene inheritance would most likely make tolerance more evolutionary stable than specific resistance. This strategy may also be more durable in crop plants (Salomon, 1999, Roy and Kirchner, 2000). In addition, considering that WDV and many other pathogens are generalists and occur frequent in wild grass populations nearby wheat fields, the evolution of new strains and races is persistent and a tolerance defence strategy may therefore be more resilient against pathogen diversity. Based on the above, tolerance should be considered as an additional breeding goal for improvement of crops. Future pre-breeding programs should thus be directed towards investigating the potential of tolerance as a durable defense strategy in crop plants, not only to single strains and races but a variety of pathogens common in the local cropping system.

6.3 Genetic resources for durable plant defense

The durability of defense towards WDV and other pathogens is likely to increase by combining genes from various genetic resources with variation in tolerance and partial resistance (Brown, 2015). Gene pyramiding by accumulating genes in the same genotype has, for example, been successful in improving the durability in resistance to stem rust in wheat (Singh et al., 2011). Another breeding strategy is to improve durability in the crop by developing cultivars with different tolerant and resistance genes and grow them in cultivar mixtures (Mundt, 2014).

To be able to accumulate genes within genotypes and cultivars, and to develop cultivars with different alleles and genes for tolerance and partial resistance the breeders need to have access to a gene pool with large enough diversity. By studying the response pattern in various accessions of the wild wheat relatives with different environmental and genetic origins we have been able to identify several tolerant and partial resistant genotypes. Due to the diverse genetic and ecological background these genotypes may have evolved different genes and alleles for tolerance and resistance to WDV and are therefore of potential interest for breeding. For continued search for potential genetic resources to expand the gene pool, our findings suggest that it is most likely to find genotypes of *Ae. tauschii* with lower WDV content and milder symptoms in the Temperate grasslands, savannas and shrublands biome than in other biomes across the range of *Ae. tauschii*. However, tolerant and partial resistant accessions have also been identified in other biomes and the search

should not be restricted to specific biomes and regions. In this way the ability of *Ae. tauschii* to adapt to diverse environments will be utilized to maximize the chance of collecting different genes and alleles for breeding of durable tolerance and partial resistance to WDV in wheat. However, using this wild gene pool it is also important to consider other agricultural important traits during selective breeding. Through crosses between wheat relatives and wheat, wild and undomesticated traits may be introduced which may affect plant growth and development, and other domestication traits such as shattering. However, the selection against shattering and other wild traits with known genetic basis will be facilitated by genetic markers (Sang, 2009). Trade-offs due to cost of resistance, for example, between defense and vegetative and sexual reproduction may also occur (Huot et al., 2014).

Knowledge about the genetic basis of tolerance and resistance to WDV will make the selection more precise and effective and the studied *Ae. tauschii* accessions are important tools to achieve this goal. Accessions with different response patterns are ideal parents in crosses for development of mapping populations in order to identifying quantitative trait loci controlling variation in response to WDV. They are also useful genotypes in differential gene expression studies for discovery of tolerance and resistance genes. The significance of these genetic methods will increase when used in combination with the released whole-genome sequence of *Ae. tauschii* (Jia et al., 2013) and bread wheat (Mayer et al., 2014).

To conclude, by providing knowledge about the diversity in host response patterns to WDV, and how that diversity is structured according to environmental and genetic origins of the wild relatives of wheat, and by identifying tolerant and partial resistant accessions useful as genetic resources in pre-breeding and breeding, the overall aim of this thesis has been achieved. As plant breeding plays a key role in sustainable agriculture and food security the findings of this thesis will add to the knowledge necessary to meet the increasing global demand of food.

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